A Strain of the Fungus *Metarhizium anisopliae* for Controlling Subterranean Termites

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ABSTRACT Alates of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, collected after swarming in 2002 died within 48 h, and the cadavers were visibly infected with a fungus. Fungi were picked from the cadavers, transferred to media, and ultimately isolated to purity. The individual fungal cultures were then used to infect Formosan subterranean termite workers. A single fungal isolate, C4-B, taxonomically identified as *Metarhizium anisopliae* (Metschnikoff), was found to cause rapid mortality of Formosan subterranean termite alates. This is the first report of a biological control agent for termite alates. In initial experiments, C4-B was more lethal to both alates and workers compared with *M. anisopliae* strain ESC 1, previously marketed as the termite biocontrol agent BioBlast. Dose–response assays in which Formosan subterranean termite alates were exposed to a known concentration of C4-B spores revealed that 10^6 spores/μl killed 100% of the alates in 3 d, both 10^5 and 10^4 spores/μl in 6 d, 10^3 spores/μl in 9 d, and 10^0 spores/μl in 12 d. Assays with workers demonstrated that 10^6 and 10^5 spores/μl killed 100% of the workers in 6 d. In an experiment to test the transfer of inoculum from infected workers to uninfected nestmates, 62.8% of the workers died in 21 d when only 20% of the workers had been inoculated. Mortality of alates caused by C4-B was tested at two field sites by dispersing fungal spores on grassy lawns and collecting alates from the treated areas. Alates thus infected showed 100% mortality by day 5, whereas only 64.8% of untreated control alates from the same collection area were dead on that day.

KEY WORDS alate, pathogenicity, dose response, field study, Formosan

SUBTERRANEAN TERMITES, including the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, cause an estimated $1 billion in prevention and control costs in the United States annually (Potter 1997). The Formosan subterranean termite is becoming the predominant termite pest species in several southern states and in Hawaii. Current Formosan subterranean termite control methods involve slow-acting, nonrepellent termiticides and baits (Su and Scheffrahn 1998). Foraging workers carry these termiticides back to the nest and spread them to nestmates, thereby reducing colony size (Su and Scheffrahn 1998). Insect pathogens are attractive candidates for baiting because of their self-replicating nature and safety to nontarget animals. According to Glare and Milner (1991), most virulent isolates are those derived from the target insect infected under field conditions. Factors that have limited the development of termite biological control agents include 1) removal of the pathogen by termites by grooming and 2) isolation of infested members of the colony (Culliney and Grace 2000).

Most studies addressing the conditions required for control in the field have focused on *Metarhizium* spp. and *Beauveria* spp. (Milner et al. 1998b; Sun et al. 2002, 2003a). Recently, Wright et al. (2003) patented *Paecilomyces* spp. for controlling subterranean termites. These *Paecilomyces* strains are nonrepellent, are transferred among termites, and cause rapid mortality. Milner et al. (1998b) tested 93 isolates of *Metarhizium anisopliae* (Metschnikoff) obtained from two species of termites. Isolate FI-610 was one of the most effective. Successful control of *Coptotermes acinaciformis* (Froggot) was achieved using direct inoculation with 3×10^{11} conidia into termite mounds in Australia (Milner et al. 1998b). Although it can be difficult to identify the location of a Formosan subterranean termite nest within a structure, infestations in trees are similar to termite mounds, thereby allowing localized access for direct treatment. It may therefore be possible to deliver the *M. anisopliae* strain described here to trees by using methods similar to those used by Milner et al. (1998b). It is anticipated that an effective biological control agent can be used alone (Grace 2003) or in a synergistic partnership with chemical termiticides (Boucias et al. 1996) for termite population management.

M. anisopliae is a well known fungal pathogen of a wide range of insect species (Hanel and Watson 1983,

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Rath et al. 1995, Jones et al. 1996, Milner et al. 1998b, Ramakrishnan et al. 1999, Staples and Milner 2000, Dean et al. 2002). An isolate of this fungus, ESC 1, has been commercialized as a mycoinsecticide (BioBlast) for use in termite control. However, when allowed to infect Formosan subterranean termite alates in this study, strain ESC 1 caused only moderate rates of mortality over relatively long periods of time. Herein, we report the isolation of *M. anisopliae* strain C4-B that causes rapid mortality among alates and workers of the Formosan subterranean termite. We also conducted a preliminary experiment to determine the efficacy of C4-B in a field test.

Materials and Methods

Collection of Termite Workers. Formosan subterranean termite workers, third instar or older based upon size, were obtained from bucket traps (Su and Scheffrahn 1986) set up at the Southern Regional Research Center, City Park, and the University of New Orleans, all of which are located in New Orleans, LA. Formosan subterranean termite workers from four different colonies of termites were chosen to prevent colony vitality biasing of data.

Collection and Identification of Fungal Strain. Alates of *C. formosanus* were collected in universal blacklight traps (BioQuip, Gardena, CA) from several locations in New Orleans between April and June, the normal swarming season. Termites were removed from the light traps within 1 h, held overnight in plastic boxes containing moist paper towels, and brought to the laboratory the next morning. In 2002, all alates from one particular collection died within 48 h. Fungal mycelia and spores were visible on the cadavers. Conidia were transferred from the dead alates onto potato dextrose agar (PDA) by using sterile transfer loops. The plates were incubated at 25°C for 4–7 d until the mixed cultures sporulated. Fungal conidia were transferred to fresh PDA and incubated for an additional 7 d at 25°C. The resulting colonies were repeatedly transferred onto fresh PDA as necessary until pure cultures were obtained. These pure cultures were incubated at 25°C until sporulation was evident. Five morphologically different fungi were recovered using this isolation procedure. To determine which strains were pathogenic to Formosan subterranean termite and whether these could be transferred from termite to termite, 10 workers were selected from each of the four colonies. These termites were placed on the surface of a single heavily sporulating culture for 5 min and then transferred to moistened filter paper in a petri dish with 10 of their uninoculated nestmates. The termites were incubated at 25°C and 100% RH. The single strain that caused significant mortality was identified as *M. anisopliae* by Dr. Maren A. Klich (USDA–ARS, New Orleans, LA) and placed into the Southern Regional Research Center Culture Collection as SRRC 2571.

Mortality Assays. In initial experiments pathogenicity of strains C4-B and ESC 1 was compared by placing 20 Formosan subterranean termite workers from each of four colonies in a (90 by 10-mm-diameter) petri dish containing filter paper moistened with water. The filter paper was covered with spores mixed with silica in a total volume of 1.5 g. Cultures of both C4-B and ESC 1 were grown for 7 d at 25 C on PDA. Spores were then harvested from the plates by scraping with a sterile spatula. An estimate of the number of spores was made using a hemacytometer (Hausser, Horsham, PA). In total, 6.5×10^{10} spores of both C4-B and ESC 1 were individually distributed over the surface of moistened filter paper in (90 by 10-mm-diameter) petri dishes. The plates were incubated at 25°C and 100% RH. Mortality was recorded daily. Each treatment was replicated twice. The test was repeated with alates collected in May 2003. In a separate set of experiments, 1:10 and 1:100 serial dilutions of spores in silica resulted in the application of 9.7×10^9 and 9.7×10^8 per plate, respectively. Groups of 20 alates were exposed as described above to undiluted spores of each strain (C4-B and ESC 1), or to 1:10 or 1:100 dilutions of C4-B applied to each petri dish. Exposure to each of the C4-B treatments was replicated 12 times, whereas exposure to ESC 1 was replicated six times.

Spore Transmission Assays. Formosan subterranean termite workers were used in this experiment. Of the 20 workers in each replicate, members of a 0, 5, 10, or 20% subset were each inoculated with a 0.5-μl aliquot of a spore suspension containing 2×10^6 spores/μl. Termites were then transferred to petri dishes containing moistened filter paper and the remaining nestmates. The plates were incubated at 25°C and 100% RH. Each treatment ratio was replicated three times. Individual colonies represented a single replicate in each experiment.

Dose Response in Alates. Dilutions of C4-B spores were made in 0.05% Triton X-100 to final concentrations of 1×10^0 through 1×10^6 spores/μl. Alates were collected in May 2004 from light traps in New Orleans and were used the day after collection. Alates were immobilized on ice, and a 1-μl aliquot of each spore suspension was applied to the ventral surface of each alate. Treated alates were left on ice for 20–30 min to allow the suspensions to dry on the surface of the insect. Groups of five alates were then transferred to (50 by 9-mm-diameter) petri dishes lined with moist filter paper and incubated at 28°C and 100% RH. Control alates were treated with 1 μl of 0.05% Triton X-100 only. Each dilution and control was replicated three times. Mortality was recorded daily.

Dose Response in Workers. The dose–response experiment was repeated for Formosan subterranean termite workers with minor modifications from the alate dose–response experiment. The termites were collected from three colonies by using bucket traps (Su and Scheffrahn 1998). The spore concentrations used were 2×10^1 through 2×10^6 spores/μl, and a dose of 0.5 μl was used per individual. The small size of workers caused them to stick to the pipette tip as the spore suspension was being dispensed, so the suspension was instead dispensed as droplets onto a sheet of glass and a chilled, immobilized worker was placed dorsal side down onto each droplet. The workers remained on the glass plate for 20 min and were then transferred in groups of 10 to (50 by 9-mm-diameter) petri dishes with moist filter paper and incubated as described above. Control workers were treated with

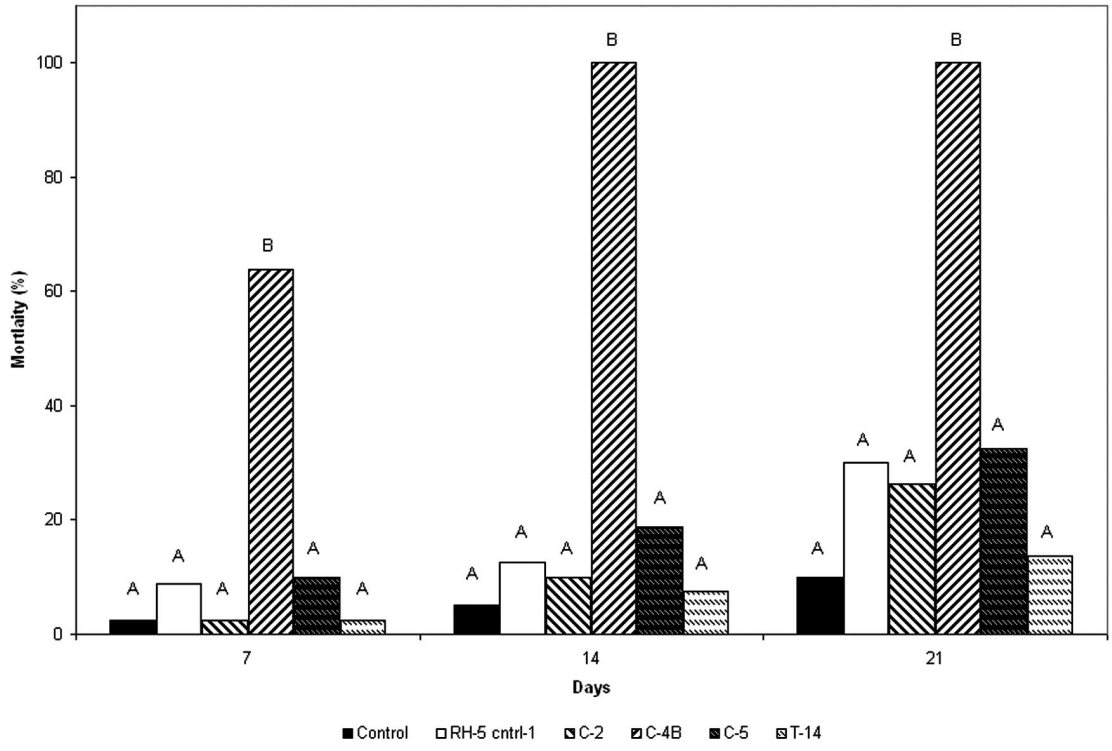

Fig. 1. Mortality of Formosan subterranean termite workers after exposure to fungal strains newly isolated from Formosan subterranean termite alate cadavers.

0.5 μl of 0.05% Triton X-100 only. Dilutions and controls were replicated three times. Mortality was recorded daily.

Field Trial and Recovery of Fungi from Termite Cadavers. Two 2 by 2-m areas of turf set 5–7 m apart were selected near known swarming sites. The borders of the test areas were marked with flags. A black light (used in light traps) was placed in the center of each of the two areas. At the earliest indication of alate swarming, one of the areas was dusted with C4-B spores mixed with corn cob grits at a rate of 1×10^{11} spores/m^2 in a total volume of 100 ml of corn cob grits. The other area was dusted with 100 ml of corn cob grits only. After 20–30 min of alate activity around the lights, a 50 by 50-cm white cloth was placed on the ground in each area to attract the swarming alates from the grass. The alates crawled onto the white cloth. The pieces of cloth from the control and treated areas, with the alates attached, were transferred to plastic boxes. On the next morning 125 alates from each area were transferred in groups of 25 into 90 by 15-mm petri dishes containing moist filter paper and incubated at 28°C and 100% RH. Mortality was recorded daily. The experiment was replicated once using alates collected from a different location. A 10% random sample of cadavers was selected for fungal recovery analysis. Individual alates were placed in separate sections of quadrant petri dishes and were refrigerated until they could be analyzed, generally no more than overnight. Half of the cadavers were washed by being placed ventral side up on Whatman filter paper and saturated with 70% ethanol, which was allowed to dry. Both washed and unwashed cadavers were incubated in individual sections of quadrant PDA plates at 25°C until growth was observed. Representative fungi, based on morphology, were transferred to whole PDA plates, incubated at 25°C until growth was observed, and serially transferred until a pure culture was obtained. The pure cultures were identified by Dr. Maren A. Klich (USDA–ARS, New Orleans, LA).

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) and least significant difference (LSD) at $P \leq 0.05$ (Cody and Smith 1997). In each experiment, treatments with the same letter on the same day are not significantly different. All analyses were run using the SAS System Software (Cody and Smith 1997).

Results and Discussion

A group of Formosan subterranean termite alates collected in a light trap in New Orleans during the 2002 swarming season showed unusually high, rapid mortality and were quickly covered with fungal mycelia and conidia. Several fungal strains isolated from the alate cadavers were purified individually. Each purified strain was tested for mortality effects against Formosan subterranean termite workers (Fig. 1). We could not immediately test these on alates because

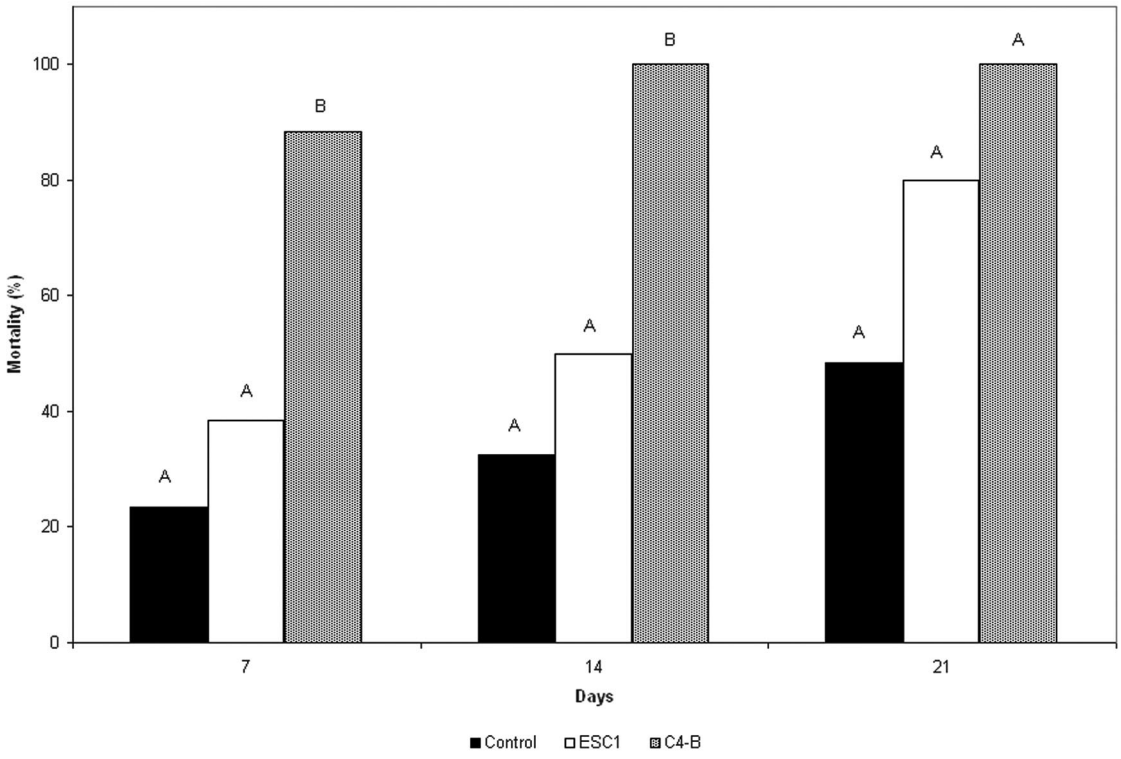

Fig. 2. Mortality of Formosan subterranean termite workers after exposure to a commercial strain (ESC 1) and a newly isolated strain (C4-B) of *M. anisopliae.*

the alate season had ended by the time the fungal strains had been purified. Of the five strains tested only one, designated C4-B, caused significant mortality among workers. By day 7, C4-B caused 63.8% mortality, whereas 100% of the termites were dead by the 14th day (Fig. 1). C4-B was identified as a strain of *M. anisopliae.*

In 2003, effectiveness of C4-B was first evaluated by exposing Formosan subterranean termite workers to either the newly identified strain or ESC 1, a strain of *M. anisopliae* produced commercially for control of termites under the product name BioBlast. Whereas 100% of the workers in the C4-B-treated group were dead after 14 d, only 50% treated with ESC 1 died during the same period (Fig. 2). To test efficiency of C4-B against alates, a dilution series of fungal spores in silica was applied to freshly collected alates. An undiluted dose of C4-B killed 100% of the exposed alates in 3 d, whereas the 1:10 and 1:100 reached maximal mortality rates of 95.8 and 20.6% mortality by day 4, respectively (Table 1). By comparison, an undiluted preparation of strain ESC 1 caused a mortality rate of only 73.3% among alates after 4 d (Table 1).

Recently, Wright et al. (2002) reported 50% mortality among Formosan subterranean termite workers, 5 to 6 d after exposure to ESC 1. Mortality of Formosan subterranean termite workers was examined when different proportions of termites were exposed to C4-B and incubated with uninfected nestmates. After 21 d, 5, 10, and 20% ratios of infected to uninfected individuals caused mortality rates of 35.0, 43.3, and 62.8%, respectively, compared with a control mortality rate of 14.5% (Table 2). Transmission of the C4-B isolate is evident by mortality of greater numbers of

Table 1. Percentage of mortality of *C. formosanus* alates caused by strains C4-B and ESC 1 (commercial strain) of *M. anisopliae* during the 2003 swarming season

Isolate	Concn	Days after treatment			
		1	2	3	4
Control	–	7.9 ± 3.5A	19.4 ± 5.7A	29.7 ± 7.1A	43.8 ± 8.2A
C4-B	Undiluted	22.9 ± 3.5B	90.6 ± 5.6B	100.0 ± 0.0B	100.0 ± 0.0B
	1:10	2.5 ± 1.4A	5.9 ± 2.5C	46.2 ± 7.6C	95.8 ± 3.7B
	1:100	0.8 ± 0.8A	2.1 ± 1.4C	4.6 ± 2.0D	20.6 ± 5.0C
ESC 1	Undiluted	21.7 ± 3.8B	55.0 ± 5.0D	65.8 ± 5.2C	73.3 ± 6.2D

Values are mean ± SE.

Table 2. Transfer of *M. anisopliae* C4-B strain spores from inoculated to uninoculated *C. formosanus* workers

% infected termites	Avg % mortality ± SD after (d)		
	7	14	21
0 (Control)	3.3 ± 1.6A	7.8 ± 0.9A	14.5 ± 2.5A
5	11.2 ± 3.8B	21.7 ± 3.3B	35.0 ± 6.0B
10	18.9 ± 1.9C	30.0 ± 4.4B	43.3 ± 8.8B
20	33.3 ± 2.9D	42.8 ± 6.3C	62.8 ± 0.9C

Values are Mean ± SE.

workers within 21 d than were originally exposed to spores of the fungus. Thus, with only 5% of the workers inoculated 35% of the total population was killed by day 21 (Table 2). Transfer of a pathogen among termites, particularly in the case of subterranean termites, will be necessary for this fungus to work successfully as a biocontrol agent in the field.

To determine the number of spores required to kill a single termite, dose–response experiments were designed as a measure of mortality from a known inoculum. Alates immobilized on ice received a single ventral application of C4-B ranging from 10^0 through 10^6 spores/μl. Spores were diluted in Triton X-100 to improve suspension of the spores and their adhesion to the surface of the termites. All alates exposed to a concentration of 10^6 spores/μl were dead by 3 d after exposure, those exposed to 10^5 and 10^4 spores/μl by 6 d, and those exposed to 10^3 spores/μl by 9 d (Fig. 3). By the ninth day treatments with 10^3, 10^2, 10^1, and 10^0 spores/μl had all reached a mortality rate of 93.3% (Fig. 3). The control mortality rate on day 9 was 53.3%. The lower mortality rate of the controls in these experiments compared with the field trial controls is possibly due to the lower number of alates per petri dish in these tests, which more closely matched the natural paired ratio of alates. Dose–response also was measured with Formosan subterranean termite workers. The delivery method was slightly modified in that the doses were pipetted onto a glass plate and the workers were immediately placed on the drop of liquid. Workers exposed to 10^6 and 10^5 spores/μl reached 100% mortality by day 6 (Fig. 4). No other treatment reached 100% mortality. The mortality in the control group was only 3.3% after 6 d (Fig. 4). Mortality caused by dilutions ranging from 10^4 through 10^1 spores/μl did not exceed 30% by day 12 of the experimental period (Fig. 4).

Field trials were performed to measure the effectiveness of strain C4-B against field-exposed alates. At each test site swarming alates were attracted to blacklights placed at the center of grassy areas inoculated either with C4-B spores mixed with corn cob grits, or corn cob grits alone (control). Mortality of alates collected from the treated area reached 93.6% in 3 d, whereas mortality of the untreated alates was only 34.0% on the same day (Fig. 5). Moreover, exposed alates showed 100% mortality in 6 d, whereas alates not

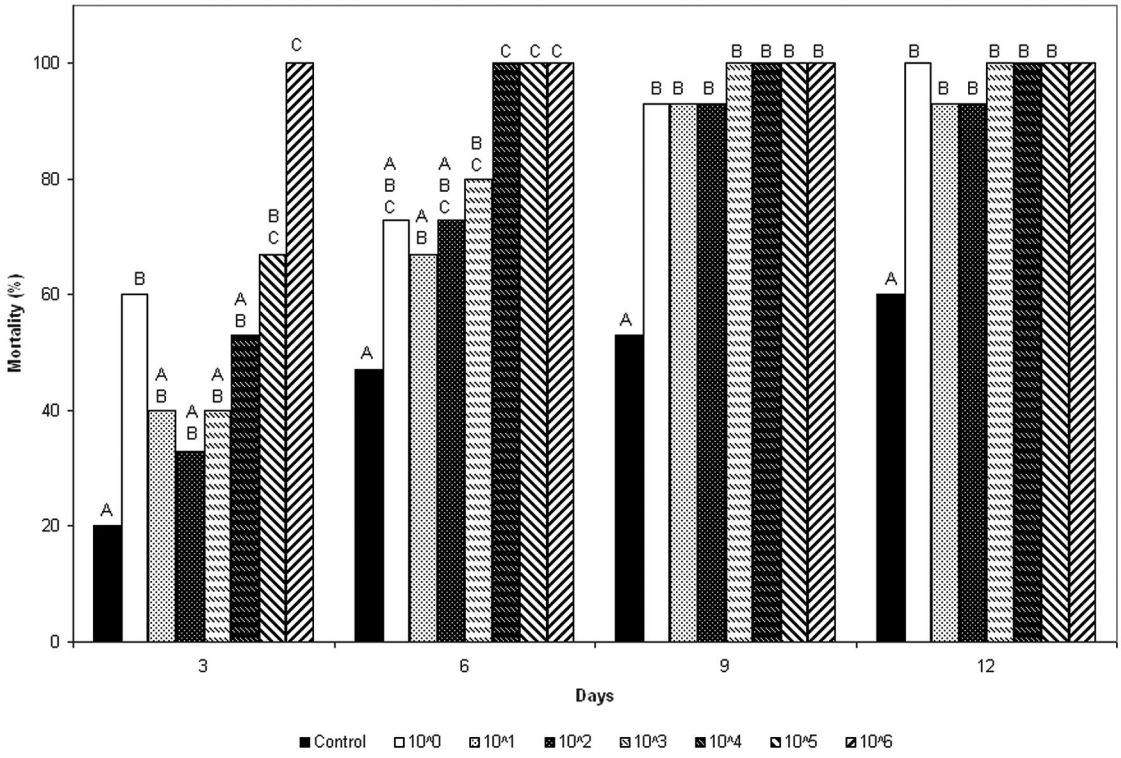

Fig. 3. Dose response of Formosan subterranean termite alates to known spore concentrations of *M. anisopliae* strain C4-B.

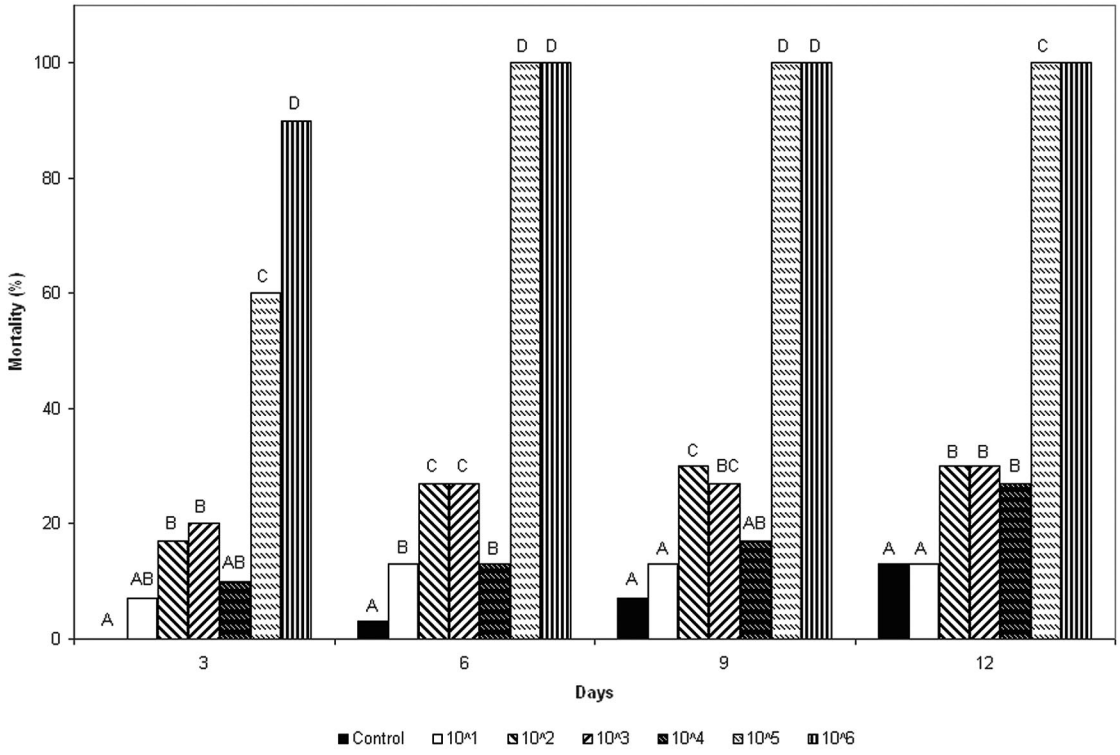

Fig. 4. Dose response of Formosan subterranean termite workers to known spore concentrations of *M. anisopliae* strain C4-B.

exposed to the fungus showed a maximum of only 64.8% mortality on day 6 (Fig. 5). It is our experience that groups of alates kept in petri dishes do not generally survive longer than 7 to 8 d. The relatively high mortality rate seen in Fig. 5 most likely reflects natural mortality of alates maintained in the laboratory. Captured alates generally experience high mortality unless they pair up and initiate a nuptial chamber (data not shown). Fungi were recovered from Formosan subterranean termite cadavers after field tests by plating the cadavers in PDA and serially purifying fungi that grew from them. Alcohol washing removed and/or killed fungal spores on the surface of the termite to allow growth of fungi inside the cadaver. Recovery yielded more fungi from termites exposed to C4-B (78% from washed and 63% from unwashed cadavers) than controls (22% from washed and 37% from unwashed cadavers). Fungal cultures presumed to be *Metarhizium* sp. by morphological comparison to a representative sample were more evenly distributed with 45% recovered from washed C4-B, 23% from unwashed C4-B, 25% from washed control and 26% from unwashed control cadavers (data not shown). It is not unusual to find *Metarhizium* spores on the surface of termites from natural exposure to the fungus in the soil. Further genetic analysis would be necessary to determine whether the newly isolated virulent strain described here is the strain present in each of the cadavers. These data show that the majority of the fungi were recovered from the termites known to have been exposed to C4-B.

It has been proposed that the natural presence in the soil of pathogenic fungi, particularly *Metarhizium* and *Beauveria*, may contribute to control of Formosan subterranean termite by killing termites before new colonies can be established (Sun et al. 2003a, b). Other studies have shown that although termite nests are generally free of fungal disease (Roberts and Humber 1981, Milner 1997, Milner et al. 1998a), the success of founding pairs in new soil is low (McMahan 1962, Rosengaus and Traniello 1993, Fei 2000). Because alates give rise to new colonies after swarming, elimination of this caste through targeted delivery of a pathogenic fungus before or during a swarm could be crucial to long-term termite management. Alates stay in a parent colony for 1 wk to several weeks before swarming. Strain C4-B is easy to grow on available media and its spores, when mixed with an inert carrier, can be introduced into termite nests as a powder to infect the maturing alates. Alternatively, spores could be broadcast to infect alates upon their dispersal flights. Additionally, the spores also kill workers, thereby reducing the colony population. High relative humidity inside a subterranean termite colony and a confined area are considered conducive to the growth and survival of pathogenic agents such as fungi (Ignoffo 1992). Existing control measures are directed primarily against Formosan subterranean termite

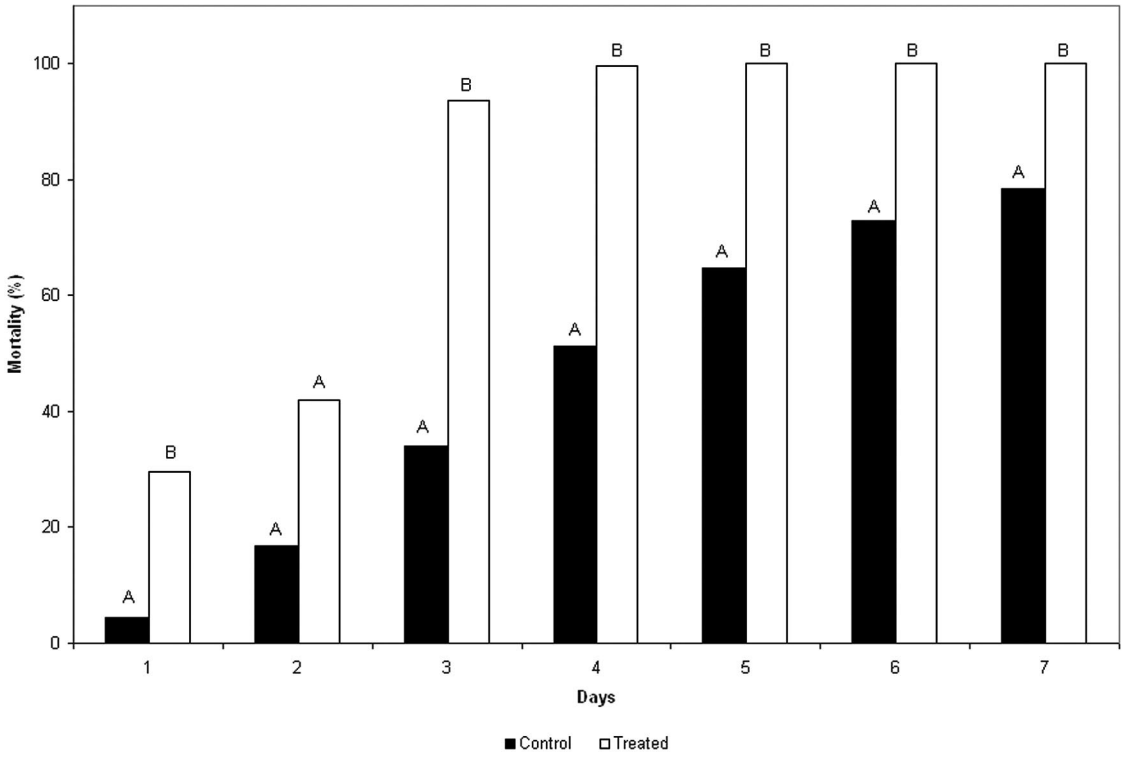

Fig. 5. Mortality of Formosan subterranean termite alates after exposure to spores of *M. anisopliae* strain C4-B in the field.

workers. Strain C4-B, although rapidly killing alates, is also lethal to workers. This strain has the potential to be produced in large quantity and formulated for control of termites in an integrated pest management system. Further research is needed to determine the optimal formulations and delivery mechanisms for termite control and to determine long-term effects on the colony in the field.

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